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**Research Article**

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# Low concentration of Cr(VI) inhibited proliferation and self-renewal of hepatic stem/progenitor cells by suppressing Wnt signaling activity

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**ABSTRACT**

*The aim of the present study was to investigate the effect of low concentration of Cr(VI) on proliferation and self-renewal of hepatic stem/progenitor cells (HSPCs) and their possible mechanism of action. WB-F344 cells, a rat-derived multipotent HSPCs line, were cultured with various doses of Cr(VI) for 24 hours or 30 days. The results showed that low concentration of Cr(VI) inhibited proliferation and self-renewal of WB-F344 cells, in which cell colony formation efficiency and colony diameter were significantly decreased in a concentration dependent manner. Microarray and qPCR results revealed that the expression of several genes belonging to Wnt signaling were downregulated. Consistent with gene expression analysis, treatment of WB-F344 cells with Cr(VI) resulted in reduction of transcriptional activator β-catenin, together with its translocation from nucleus into cytoplasm. Our finding suggested that low concentration Cr(VI) inhibited proliferation and self-renewal activity of WB-F344 cells by suppressing Wnt signaling activity.*

**Key words:** Hexavalent chromium; Hepatic stem/progenitor cells; Self-renewal; Wnt signaling

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## INTRODUCTION

Adverse health effects of Cr(VI) exposure include lung cancer, gastrointestinal symptoms, hypotension, and renal failure[1,2]. Based on the findings in animals, the liver was likely to be an important site of cellular uptake of Cr(VI), and increased levels of chromium with dose had been observed in the liver of mice. Correspondingly, studies demonstrated that Cr(VI) were associated with liver damage such as hepatocytes necrosis and apoptosis[3,4]. Stem/progenitor cells are of interest clinically because of their potential to replenish or repair damaged tissue. Recent studies had indicated that low concentration of heavy metal ions would affect proliferation, self-renewal and differentiation of stem cells. Jiang et al. described that cadmium inhibited proliferation and self-renewal acitivity of prostate stem/progenitor cells by suppressing androgen receptor[5]. Tamm et al. reported that exposure to MeHg at concentration comparable to the current developmental exposure of general population inhibited spontaneous neuronal differentiation of neural stem cells[6]. Chen et al. found that submicromolar concentration of Cr(VI) strongly inhibited contracting cardiomyocyte development in wild-type embryonic stem cells by activation of MAP2K4/7-dependent JNK and MAP2K4-dependent p38[7].

Hepatic stem/progenitor cells (HSPCs), located in the canal of Hering, were demonstrated to have capacity to regenerate injuried liver by differentiation into hepatocytes and cholangiocytes[8]. In the present study, we investigated the effect of low concentration of Cr(VI) on WB-F344 cells, a rat-derived multipotent HSPCs line with well studied[9,10]. We focused our attention on Cr(VI) induced change of proliferation and self-renewal capacity of WB-F344 cells, as well as their possible mechanism of action.

## EXPERIMENTAL SECTION

### Cell Culture

The rat pluripotent HSPCs-like cell line, WB-F344 cells, were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences and were cultured in DMEM (Invitrogen, CA) supplemented with 10% FBS (Hyclone, UT), 100 U/mLpenicillin (Invitrogen, CA), and 100 mg/ml streptomycin (Invitrogen, CA). The cells were cultured at 37°C in an incubator with a humidified atmosphere containing 5% $CO_2$. Media were changed every other day, and cells were split every 4-5 days.

### Determination of cellular reactive oxygen species (ROS)

To determine intracellular accumulation of ROS, the WB-F344 cells were treated with different concentration ( 0, 0.1, 0.5, 1, 5, 10, and 50 μmol/L) of Cr(VI) for 24 hours. The membrane permeable indicator dihydrodichlorofluorescein diacetate (H2DCF-DA) Kit (Jiancheng, China) was used according to the manufacturer's directions. Suspended cells with H2DCF-DA staining were monitored with an inverted fluorescence microscope (Nikon, Japan). Reactive oxygen species were determined by comparing the changes in fluorescence intensity with those of the histogram.

### Colony formation and colony diameter

WB-F344 cells were pre-treated with low concentration (0, 0.1, 0.5, and 1μmol/L) of Cr(VI) for 24 hours or 30 days. After then, control and Cr(VI)-treated cells were plated at 100 cells/dish in 10-mm cell culture dishes, and cultured for 7 days in the absence of Cr(VI). The Cells were stained with Giemsa solution, and the number of colonies was then counted. Photographs were taken at representative areas in culture plates. The diameters of individual colonies were measured using Zeiss LSM image Examiner software. At least 20 representative colonies were measured from each independent experiment.

### Microarray and Quantitative polymerase chain reaction (qRT-PCR)

WB-F344 cells were treated with 0.5 μmol/L of Cr(VI) for 24 hours or 30 days. After then, microarray analysis was conducted using Agilent Rat Whole Genome 8×60 k Arrays to analyze ~40 000 transcripts. Gene expression data were ranked and prioritized using |fold change|>2 criteria to identify differentially expressed genes. Samples of total RNA were isolated from cells used in microarray hybridization using SYBR Green-based qRT-PCR. RNA was reverse transcribed using AMV reverse transcriptase. 0.5 μL cDNA solution was used in a 20-μl PCR reaction containing 10 μL SYBR premix Ex Taq, 0.4 μL Rox (Takara, Japan), 10 μmol/L forward and reverse primers. Primer sequence for Lef1 is 5′-GCAAGGTCAGCCTGTTTA-3′/5′- GGCCCAGGAATCATGTG-3′; for Wisp2 is 5′- AGACAGCAGATTGCCTGAAA-3′/5′- AGGTCAGGGAAGGTACACTTA-3′; for β-catenin is 5′-TCCTGCACCACCAACTGCTTAG-3′/5′-AGTGGCAGTGATGGCATGGACT -3′. The amplicons were approximately 100–200 bp. The PCR amplifications were conducted in 96-well BIOplastics (Axygen, USA) on an Applied Biosystems PRISM 7300 Sequence Detection System under the following conditions: 10 seconds denaturation and enzyme activation at 95 °C, followed by 40 cycles of denaturation (95 °C, 10 seconds), and annealing (60 °C, 30 seconds). Results were normalized to β-actin to control for differences in RNA loading, quality and cDNA synthesis. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. Each sample was assayed in triplicate and the median threshold cycle values were used to calculate the fold change between treated and control samples.

### Western blot and immunocytochemistry

Western blot and immunocytochemistry were performed by traditional methods as previously decribed[11]. Primary antibodies against β-catenin (rabbit anti-human, mouse, rat, and monkey) (Cell Signaling Technology, USA) and β-actin (Cell Signaling Technology, USA) and appropriate peroxidase-conjugated secondary antibody (Dako EnVsionTM+ System, HRP) were used according to the manufacturer's directions.

### Statistical Analysis

All data were expressed as Standard Error. Statistical comparisons were performed using Student's t-test ($P < 0.05$ was considered statistically significant).

## RESULTS AND DISCUSSION

### Low concentration of Cr(VI) did not cause acute cytotoxicity in WB-F344 cells

In order to assess the acute cytotoxicity effect of Cr(VI) on WB-F344 cells, the cells were treated with different concentration (0, 0.1, 0.5, 1, 5, 10, and 50 μmol/L) of Cr(VI) for 24 hours. The cell morphlogy and oxidative stress were then evaluated. As shown in Fig.1A, cells both in the control and in the 0.5 μmol/L of Cr(VI) treatment group retained their the typical epithelial cell morphology with flat and diamond-shape as previously described[9,10].

However, in the 50 μmol/L of Cr(VI) treatment group, the cells shrunk and became to apoptosis. Cr(VI) is known as an inducer of ROS and high amounts ROS induced by Cr(VI) would lead to cell death or apoptosis. Therefore, experiments were carried out to detect the expression levels of ROS in the cells with or without Cr(VI) treatment. As expected, the cells with 50 μmol/L of Cr(VI) treatment were clearly present of fluorescence of H2DCF-DA (Fig.1B), indicating high amount of ROS were produced in the cells. In contrast, control cells and cells with 0.5 μmol/L of Cr(VI) treatment almost did not detect fluorescence of H2DCF-DA (Fig.1B). Histogram of fluorescence intensity of H2DCF-DA further showed that, similar to control cells, treatment of WB-F344 cells with ≤ 1 μmol/L of Cr(VI) produce very low amounts of ROS (Fig.1C). Thus, our observation demonstrated ≤ 1 μmol/L of Cr(VI) did not cause acute cytotoxicity in WB-F344 cells.

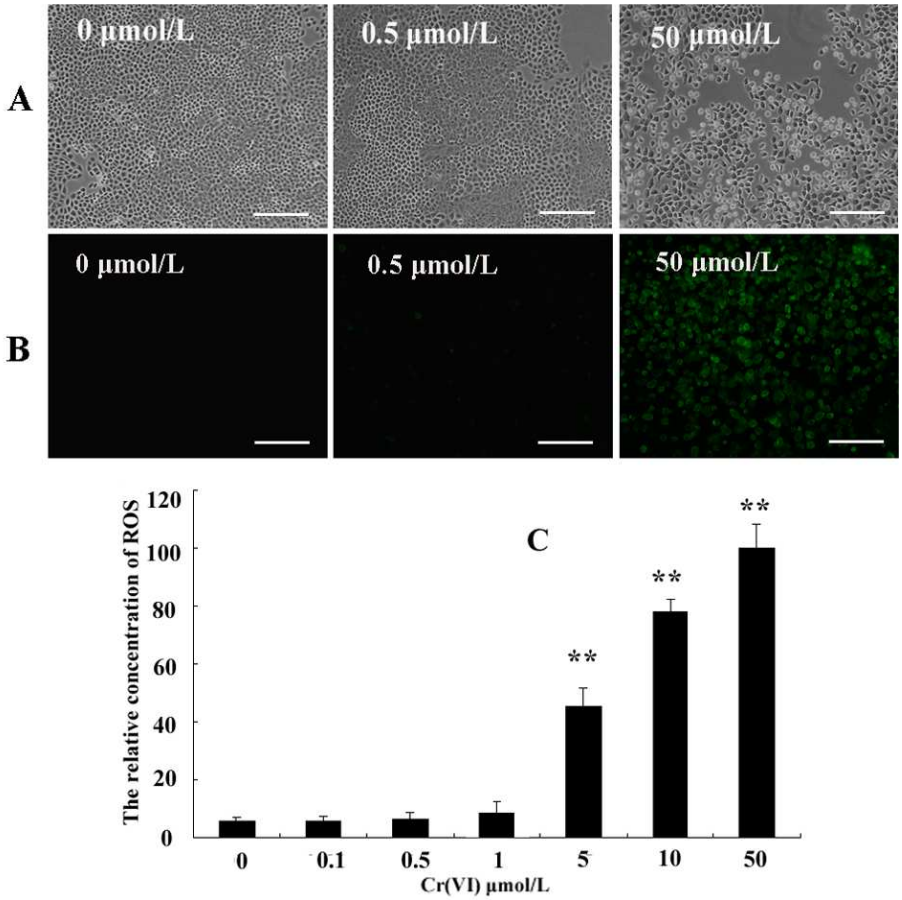


**Fig.1 Morphology and detection of ROS of WB-F344 cells after Cr(VI) treatment**

(A) Representative phase contrast images of WB-F344 cells treatment with or without Cr(VI). (B) Fluorescence intensity of H2DCF-DA (Green) was used for detection of ROS. Noted that treatment cells with 0.5 μmol/L of Cr(VI) were absence of fluorescence (Green) of H2DCF-DA. (C) Accoding to fluorescence intensity of H2DCF-DA, histogram was made. Histogram revealed that ≤ 1 μmol/L of Cr(VI) produce very low amounts of ROS, but ≥ 5 μmol/L of Cr(VI) significantly increased the production of ROS. ** P ＜0.01. (A): Scale bars: 100 μm; (B): Scale bars: 50 μm.

**Low concentration of Cr(VI) inhibited proliferation and self-renewalal activity of WB-F344 cells**

We then ask if low concentration of Cr(VI) would affect the proliferation and self-renewal activity of WB-F344 cells. WB-F344 cells were pre-treated with low concentration (0, 0.1, 0.5, and 1μmol/L) of Cr(VI) for 24 hours or 30 days. After then, control and Cr(VI)-treated cells were plated at 100 cells/dish in 10-mm cell culture dishes, and cultured for 7 days in the absence of Cr(VI) to count the number and the size of colonies. After 7 days of culture, epithelial colonies were formed (Fig.2A). Compared to the control groups, low concentration of Cr(VI) inhibited colony formation capacity in a concentration-dependent manner both in the 24 hours and 30 days treatment groups (Fig.2B), indicating that low concentration of Cr(VI) suppressed self-renewal of WB-F344 cells. Treatment of the cells with 1 μmol/L of Cr(VI) for 30 days resulted in the lowest cloning efficiency, and cloning efficiency in this group was 58% of control group (Fig.2B). As for the size of colonies, the results also showed that the diameter of colony was significantly decreased after Cr(VI) treatment (Fig.2C), indicating that low concentration of Cr(VI)

inhibited proliferation of WB-F344 cells. Treatment of the cells with 1 μmol/L of Cr(VI) for 30 days resulted in a small-colony morphology, and diameter of colony in this group was 65% of control group (Fig.2C). Thus, our data demonstrated that low concentration of Cr(VI) inhibited proliferation and self-renewal activity of WB-F344 cells especially after long-term exposure.

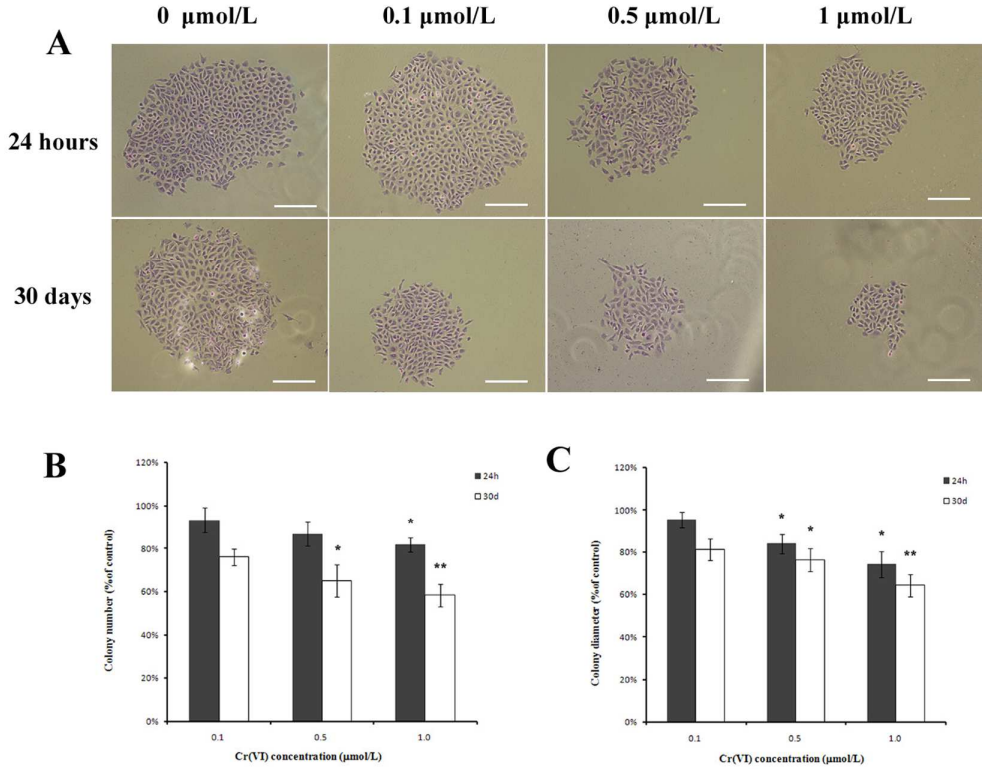


**Fig.2 Low concentration of Cr(VI) inhibition self-renewal and proliferation activity of WB-F344 cells.**

(A) Representative colony images of WB-F344 cells treatment with 0, 0.1, 0.5, and 1μmol/L of Cr(VI) for 24 hours or 30 days. (B) Cloning efficiency (compared to control cells) of WB-F344 cell was gradually reduced with the increase of Cr(VI) concentration and exposure time. (C) The diameter of colony (compared to control cells) is markedly reduced with the increase of Cr(VI) concentration and exposure time. *P ＜0.05 and ** P ＜0.01. (A): Scale bars: 100 μm.

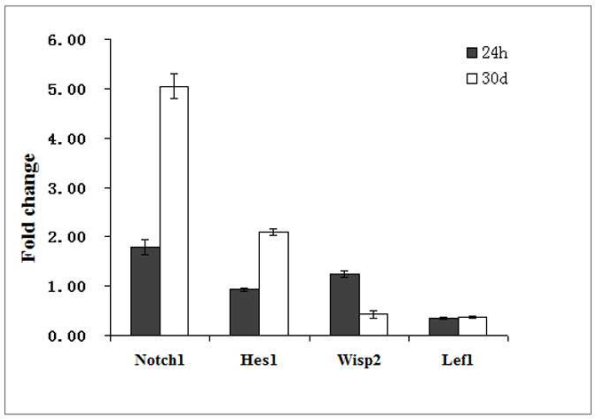


**Fig.3 Comparisons between Q-ploymerase chain reaction (qPCR) gene expression patter and microarray data between 0.5 μmol/L Cr(VI) treated and control samples.**

**Changes in genes associated with Wnt and Notch signaling by microarray and qRT-PCR analysis**

To elucidate the possible mechanisms by which Cr(VI)-induced decrease of self-renewal and proliferation of WB-F344 cells, gene chip were used to screen differential gene expression between control and treatment group. In comparison to control cells, the expression level of 685 genes were changed in the 24 hours treatment group, and

969 genes were changed in the 30 days treatment group (fold ＞ 2), respectively. Among these differentially expressed genes, we noticed that the expression level of genes belonging to Wnt and Notch signaling were changed. The expression of Notch signaling pathway related genes such as Notch 1, contactin 6 (Cntn6) and Hairy and enhancer of split 1 (Hes1) were upregulated 2.0, 1.37 and 3.08 times respectively after 24-hours exposure, and 2.73, 2.5 and 4.18 times respectively after 30 day exposure. In contrast, Wnt targeted downstream genes such as lymphoid enhancer factor (Lef1), SP5 and Wnt-1-inducible signaling pathway protein-2 (WISP2) were downregulated 11.4, 3.61 and 1.19 times respectively after 24 hours exposure, and 11.5, 6.81 and 2.6 times respectively after 30 days exposure. qRT-PCR was performed to validate the results obtained from our microarray study. As shown in Fig.3 the upregulated or downregulated patterns for Notch1, Hes1, Lef1 and Wisp2 obtained from qRT-PCR were similar to those in the microarray study.

**β-catenin expression and it's subcelluar localization**

There is accumulating evidence that Wnt/β-catenin critically regulates hepatic progenitor cell proliferation, and over-expression or inhibition of β-catenin either increases or decreases the overall liver size, respectively[12]. In view of key role of Wnt/ β-catenin in hepatic stem/progenitor cells proliferation, our attention were focused on whether treatment of WB-F344 cells with Cr(VI) influences β-catenin (a key component of the Wnt pathway) expression and it's localization in the cells. As shown in Fig.4A, treatment of WB-F344 cells with Cr(VI) revealed a slight decrease in β-catenin protein level by western blot analysis with a concentration-dependent manner. To prove whether Cr(VI) treatment has any influence on the subcellular location of β-catenin, immunocytochemistry was performed. In the control group, nearly 50% of cultured cells were strongly positive for nuclear staining of β-catenin (Fig.4B). In the Cr(VI) treatment group, especially treatment with 0.5 and 1 μmol/L of Cr(VI), almost no cells were positive for nuclear staining of β-catenin (Fig.4B), suggesting Cr(VI) treatment is responsible for the reduction of β-catenin and its translocation from nueclei into cytoplasm. Both conrtrol cells and Cr(VI) treated cells were moderately positive for cytoplasm staining of β-catenin. It is well known that Wnt/β-catenin signaling was activited when β-catenin translocated from cytoplasm into nueclei. Therefor, our data suggested that the activity Wnt/β-catenin signaling was suppressed in WB-F344 cells after Cr(VI) treatment.

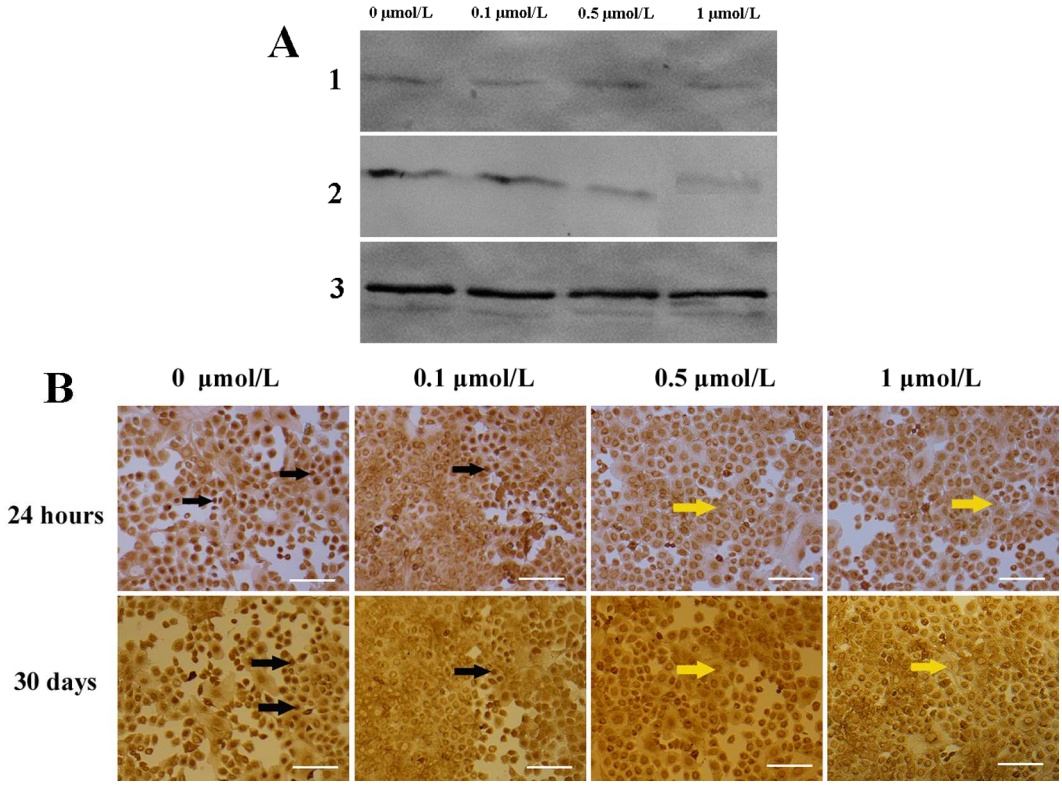


**Fig.4 Effects of Cr(VI) on β-catenin expression and its subcellular location**

(A) Treatment of WB-F344 cells with Cr(VI) revealing a slight decrease in β-catenin protein level with the increase of Cr(VI) concentration as shown by Western blot. Lane 1: Cr(VI) treatment for 24 hours, Lane 2: Cr(VI) treatment for 30 days; Lane 3: positive β-actin. (B) The number of cells with clear nuclear staining (black arrow) for β-catenin was decreased after treatment with Cr(VI). In the control group, nearly 50% of cultured cells were strongly positive

for nuclear staining (black arrow) of β-catenin. Treatment with 0.5 and 1 μmol/L of Cr(VI), almost no cells were positive for nuclear staining (yerrow arrow) of β-catenin. (B): Scale bars: 50 μm.

## CONCLUSION

This is the first example to our knowledge of evaluation the effect of low concentration of Cr(VI) on proliferation and self-renewal and their possible mechanism. The present study demonstrated clearly that low concentration of Cr(VI) inhibited proliferation and self-renewal activity of WB-F344 cells especially after long-term exposure. Furthermore, microarray, qPCR and β-catenin analysis indicated that suppression of Wnt/β-catenin signaling activity may be responsible for Cr(VI) induced reduction of cell self-renewal and proliferation ability. However, the detailed mechanism of Cr(VI) and Wnt/β-catenin pathway underlying the proliferation and self-renewal of WB-F344 cells need further study.

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